Uchl1 and its associated proteins were involved in spermatocyte apoptosis in mouse experimental cryptorchidism

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Abstract: Uchl1 was found to be involved in spermatocyte apoptosis. The aim of the present study was to test whether Uchl1 and its associated proteins Jab1 and p27kip1 were involved in spermatogenic damages in response to heat-stress in cryptorchidism. Hematoxylin and eosin (HE) staining and DNA end labeling (TUNEL) were used to observe morphological and apoptotic characteristics of spermatogenic cells; Immunohistochemical analysis was used to detect changes of Uchl1 and its associated proteins Jab1 and p27kip1 in response to heat-stress from cryptorchidism leading to spermatocyte losses; And protein affinity analysis (pull-down) and immunofluorescence co-localization were used to verify the relevance among the three proteins in spermatocytes. The results showed that, Jab1 and p27kip1, in parallel to Uchl1, increased in spermatocytes of apoptotic appearances in response to heat-stress, but not in multinucleated giant cells; Jab1 bound to Uchl1 in testis protein extracts, and co-localized with Uchl1 and p27kip1 specifically in spermatocytes with apoptotic appearances. These results suggest that the accumulation of Uchl1 protein is involved in the heat-stress-induced spermatocyte apoptosis through a new pathway related with Jab1 and p27kip1, but not the formation of multinucleated giant cells.

Key words: apoptosis; cryptorchidism; heat-stress response; spermatocyte; Uchl1

Uchl1及其关联蛋白参与小鼠实验性隐睾中精母细胞凋亡

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摘 要：以往研究显示Uchl1参与调节生理状况下小鼠精母细胞的凋亡。本文以小鼠实验性单侧隐睾为研究模型，以切除单侧睾丸和假手术作为对照，用苏木精-伊红(HE)染色和DNA末端标记(TUNEL)观察生精细胞的形态和凋亡情况；用免疫组化分析Uchl1及其相关蛋白Jab1和p27kip1在隐睾症的热应激反应导致精母细胞损失过程中的变化情况，并用亲和分析(pull-down)和免疫荧光共定位检测三种蛋白在精母细胞的关联性。结果显示，Jab1和p27kip1，与Uchl1平行，在具凋亡形态的精母细胞中响应热应激而含量增加，而在多核巨细胞中无类似变化。Jab1可以与睾丸蛋白提取物中Uchl1结合，并与Uchl1和p27kip1特异性地在具凋亡形态的精母细胞中共定位。以上结果提示，Uchl1蛋白的积累参与隐睾中热应激诱导的生精细胞凋亡过程，但不影响接下来的多核巨细胞的形成，且Uchl1蛋白的作用机制涉及Jab1和p27kip1参与的一种新途径。

关键词：细胞凋亡；隐睾；热应激反应；精母细胞；Uchl1

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PGP9.5 (protein gene product 9.5) was found in the brain of human, bovine, sheep, rabbit, rat, guinea-pig, chicken, trout, and frog, so it could have evolved from at least 400 million years ago and have been highly conserved throughout evolution [1]. For PGP9.5 has ubiquitin carboxyl-terminal hydrolase activity [2, 3], it was also named ubiquitin carboxyl-terminal hydrolase L1 (Uchl1) more commonly in recent years, especially in mammals. In addition to its abundant distribution in the brain, Uchl1 is high-level expressed specifically in gonads. In the testis, Uchl1 was expressed in gonocytes at 14 days of gestation of C57BL/6J mice [5], but on postnatal days 8 and 16, Uchl1 was localized strictly to the spermatogonia, whereas on postnatal day 30 and in adults it appeared not only in spermatogonia, but also in Sertoli cells [5]. In the testis of 25-week old mice, Uchl1 immunoreactivity was detected homogenously in the cytoplasm of both spermatogonia and Sertoli cells [6]. So, we concluded that Uchl1 gene expression will be stopped and its protein disappeared in the spermatogenic cells along in the meiotic process.

Uchl1-dependent apoptosis was demonstrated to be crucial for spermatogonial cell growth and sperm maturation[7]. Uchl1 can affect the normal spermatogenesis by modulating spermatogenetic cell apoptosis during prepubertal development [8]. But Kwon et al. reported otherwise that Uchl1 might function in mitotic proliferation in spermatogenesis because it was expressed mainly in spermatogonia and Sertoli cells. [9]. However, the spermatocytes were the main type of germ cells undergoing apoptosis in cryptorchidism [10]. For no reported immunohistochemical study has showed that Uchl1 occurs in spermatocytes, so evidence is still needed to tell whether Uchl1 affects apoptosis of spermatocytes only indirectly through Sertoli cells.

Cryptorchidism did not induce a significant increase in spermatogenetic cell apoptosis in gad mice with Uchl1 genic defect, as in the wild-type [8], which was explained by the fact that Uchl1 promoted apoptosis by the maintenance of monoubiquitin pool [11]. As for the mechanisms, a seemingly reasonable explanation was reported that the monoubiquitin promoted the ubiquitin-dependent degradation of inhibitors of apoptosis [8], but why does it not affect the inducers or activators of apoptosis simultaneously through the same pathway? So the explication above for the Uchl1 implication to the heat-stress-induced spermatogenic cell apoptosis needs more experimental supports.

Uchl1 binds to Jab1 in tumor cells [12], and the Jab1 relates to the nucleus-cytoplasm translocation of cyclin-dependent kinase inhibitor p27kip1 [13–15], but no study has reported that the Jab1 expresses in the testis, and Jab1 or p27kip1 relates to the cryptorchid testes or heat-stress. In the adult mouse and human testes, p27kip1 was found only expressed in Sertoli cells, but experiments on knockout mice indicated that p27kip1 has a role in the regulation of spermatogonial proliferation and apoptosis, and the onset of the meiotic phase in preleptotene spermatocytes [16]. The reports about the importance of p27kip1 suggest that some facts are still to be discovered especially in relation to Uchl1. Thus, the unilateral experimental cryptorchid model was used in this study to verify the association of Uchl1, Jab1 and p27 proteins in the spermatocytes, and to further explain the mechanism that Uchl1 has a role in the apoptosis of spermatogenic cells.

1 MATERIALS AND METHODS

1.1 Preparation of unilateral experimental cryptorchidism

Two-month-old male C57BL/6J mice were purchased from the animal center of Chinese Academy of Sciences in Shanghai. In a standard animal facility, the mice were fed in routine under controlled temperature at 25 °C and photoperiod of 14L:10D. Thirty animals were divided into 3 groups. For the operation group, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium at a dose of 1 mg per 25 g body weight. To rule out the possible effect by the testicular hormone decline in cryptorchidism, the mice that received the elimination of right testis and with the left testis remained were taken as control (Unilateral testicular control with one testis removal: marked as Uni-S). To make the unilateral cryptorchid model, the right testis (Cry-C) was fixed in the abdominal cavity by suturing its capsule to the abdominal wall, and the left testis (Cry-S) was kept free into the scrotum. In another control group (Con-S), sham-operation in right side testis was performed. The animals were sacrificed at different time points after surgery, then the testes were removed, one part of testes was frozen in liquid nitrogen and stored at −80 °C to obtain protein extracts, and the other part was fixed in the Bouin’s solution for immunohistochemical analysis. Three independent repeated experiments were carried out as described above.
1.2 DNA end labeling of tissue sections (TUNEL methods)

After being fixed overnight in Bouin’s solution, and embedded in paraffin wax, the cryptorchid or scrotal testis samples were sectioned in 5-μm thickness. To identify the apoptotic cells, the ApopTag® Peroxidase In Situ Oligo Ligation (ISOL) Kit (Chemicon International, Inc., Cat #S7200.) was applied according to the supplied protocols. Endogenous peroxidase was inactivated by 3% H₂O₂ in methanol. The sections were permeabilized by incubation with proteinase K solution for 10 min at room temperature. Biotinylated nucleotides are incorporated into 3-OH ends of the fragmented genomic DNA by T4 DNA Ligase Enzyme in a humidified chamber at 16 °C for 16 h. To exclude nonspecific reaction, T4 DNA Ligase Enzyme was omitted in parallel as the experimental control. Washed in PBS containing 0.05% Tween-20 for three times, the biotin-labeled DNA was recognized by streptavidin-horseradish peroxidase conjugate, stained in diaminobenzidine (DAB) reaction solution at room temperature for 5 min. The sections were counterstained slightly with hematoxylin, and dehydrated and mounted. The apoptotic spermatocytes (AC) were counted from 10 tubule cross-sections of each specimen under 400 amplification using Olympus BH-2 microscope. Meanwhile, hematoxylin and eosin (HE) staining was performed as usual to distinguish the cytoplasm of multinucleated giant cells (MC). MC was observed and counted as AC.

1.3 Detection of Jab1 binding to Uchl1 in testicular protein extracts

Frozen tissues were thawed in ice-cold protein extraction buffer (EB buffer: 50 mmol/L HEPES pH 7.5, 100 mmol/L NaCl, 10 mmol/L MgCl₂, 25 mmol/L β-glycerophosphate, 1 mmol/L Na₂VO₄, 50 mmol/L NaF, 1 mmol/L EDTA, 0.5 mmol/L EGTA; 10 μg/mL each of soybean trypsin inhibitor, leupeptin, and aprotinin, and 1 mmol/L PMSF). The tissues were homogenized in a Dounce homogenizer and centrifuged at 14 000 g for 10 min at 4 °C to obtain supernatant as total protein extracts. Protein concentrations in the extracts were evaluated by Bradford assay.

500 μL testis protein extract (1 mg total protein) was incubated for 2 h at 4 °C under constant rotation with 50 μL Sepharose CL-6B, which was pre-washed with EB buffer. After a centrifugation (1 000 g, at 4 °C for 2 min), the supernatant was removed and incubated with 20 μL glutathione Sepharose 4B beads (Amersham Pharmacia Biotech Company) binding to glutathione S-transferase (GST), under constant rotation for another 2 h at 4 °C before obtaining the supernatant. The supernatant was incubated with glutathione Sepharose 4B beads binding to GST-Uchl1 fusion protein [17] for 4 h at 4 °C. The beads absorbed with testis proteins were washed three times in EB buffer and then eluted in 1 × SDS-PAGE sample buffer. To determine whether Jab1 binds specifically to Uchl1, the proteins absorbed by GST and GST-Uchl1 fusion proteins were separated by SDS-PAGE respectively and detected by Western blotting for Jab1. The protein blots were reacted with anti-Jab1 (1 : 200 dilution, Santa Cruz Biotechnology, Inc. Dallas, USA) rabbit polyclonal antibodies. The immuno-reactive complexes on the blotted nitrocellulose membranes (immobilon™-NC, Millipore Corporation, Billerica.) were visualized by ECL method using PhosphaGLO™ AP Substrate kit (KPL, Gaithersburg, MD, USA).

1.4 Immunohistochemistry

The serial 5 μm-thickness sections were mounted on poly-L-Lysine (Sigma)-coated glass slides. Tissue sections were incubated with 10% normal bovine serum in PBST (PBS, pH7.4, containing 0.05% Tween-20), pH 7.5 for 1 h to block the nonspecific binding. Uchl1, Jab1 and p27kip1 antigens in tissue sections were recognized respectively by rabbit anti-Uchl1 (at a dilution of 1 : 400, Chemicon International Company), rabbit anti-Jab1 (1 : 50, Santa Cruz Biotechnology, Inc. Dallas, USA) and rabbit anti-p27kip1 (1 : 200, Abcam, Cambridge, UK) overnight at 4 °C. After washing for 3 times in PBST, the sections were incubated with biotin-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology, Inc. Dallas, USA) for 1 h at room temperature. After washing, streptavidin-HRP was added to bind to biotin. DAB was used as substrate for the peroxidase reaction. Sections were counterstained with hematoxylin. As a control, the primary antibodies were neutralized by the blocking peptides (16 μg recombinant GST-tUCH for per μg primary polyclonal antibody against Uchl1, expressed in our lab; 8 μg peptide for per μg primary polyclonal antibody against Jab1 or p27kip1) (Santa Cruz Biotechnology, Inc. Dallas, USA).

For indirect immunofluorescent co-localization, rabbit anti-Uchl1 and goat anti-Jab1 (Santa Cruz Biotechnology, Inc. Dallas, USA), or rabbit anti-p27kip1 and goat anti-Jab1, were mixed together to add on the tissue sections as the primary antibodies. FITC conjugated
bovine anti-rabbit IgG and rhodamine conjugated donkey anti-goat IgG were mixed as the secondary antibodies. After the sections were mounted in 10% glycerol in PBS, the green and red fluorescence were examined under microscope (Nikon 50i) in turn, with the excitation of blue and green light respectively.

1.5 Statistical analysis
The data were processed by SPSS13.0 (SPSS Inc., Chicago, IL, USA). All the data were expressed in mean ± SD, and results were analyzed for statistical significance using one-way analysis of variance (ANOVA) or t-test as indicated. The significance level was set at 0.05.

2 RESULTS

2.1 The morphological observation of unilateral cryptorchid testes
To evaluate effects of abdominal hyperthermia on the testis, we compared the changes with time between different groups. Weights of abdominal testes (Cry-C) decreased significantly ($P < 0.01$, ANOVA) with the time after the operational injury (Table 1), but no significant changes ($P > 0.05$, ANOVA) with time were found for scrotal testes. But by day 14 after operation, weights of abdominal testes (Cry-C) were reduced to about one third of controls; and at days 7, 10 and 14, they were all significantly smaller than scrotal testes as indicated in Table 1 ($t$-test).

We carefully examined the HE-stained and TUNEL-labeled slides and found that apoptotic spermatocytes in unilateral cryptorchidism group occurred at 7 day time-point in a significantly greater quantity, which decreased with the time, compared with those in the Con-S; and MC were found in the tubules only at 10 and 14 day time-points (Table 2). The morphologically apoptotic spermatocytes were observed to take on a similar appearance as reported by others [18], which showed condensation of both the nucleus and cytoplasm and a gap around the cell, but MC mostly showed

<table>
<thead>
<tr>
<th>Days after operation</th>
<th>Uni-S</th>
<th>Cry-S</th>
<th>Cry-C</th>
<th>Con-S</th>
<th>t-test between Cry-C and Cry-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>94.26 ± 6.55</td>
<td>92.84 ± 8.08</td>
<td>66.42 ± 8.23</td>
<td>101.25 ± 8.02</td>
<td>$P = 0.001$</td>
</tr>
<tr>
<td>10</td>
<td>92.35 ± 0.64</td>
<td>86.73 ± 9.77</td>
<td>49.43 ± 4.98</td>
<td>75.50 ± 0.71</td>
<td>$P = 0.000$</td>
</tr>
<tr>
<td>14</td>
<td>97.55 ± 10.82</td>
<td>85.09 ± 4.72</td>
<td>31.73 ± 6.93</td>
<td>85.93 ± 13.66</td>
<td>$P = 0.000$</td>
</tr>
</tbody>
</table>

Uni-S are testes of the unilateral testis removal group on 7, 10 and 14 days after the operation; Cry-S are scrotal testes of the unilateral cryptorchidism group on 7, 10 and 14 days after the operation; Cry-C are abdominal testes of the unilateral cryptorchidism group on 7, 10 and 14 days after the operation; Con-S are testes of the sham-operation with no significant difference between the day points. The testis weights in mg were shown as mean ± SD. Note: By $t$-test of random experimental design, doubly truncated to obtain $P$ value.

<table>
<thead>
<tr>
<th>Group</th>
<th>Apoptotic spermatocytes/tubule section</th>
<th>Multinucleated giant cells/tubule section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uni-S7</td>
<td>0.12 ± 0.12</td>
<td>0</td>
</tr>
<tr>
<td>Uni-S10</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Uni-S14</td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>Cry-S7</td>
<td>0.27 ± 0.24</td>
<td>0</td>
</tr>
<tr>
<td>Cry-S10</td>
<td>0.23 ± 0.24</td>
<td>0</td>
</tr>
<tr>
<td>Cry-S14</td>
<td>0.25 ± 0.21</td>
<td>0</td>
</tr>
<tr>
<td>Cry-C7</td>
<td>3.60 ± 3.00</td>
<td>0</td>
</tr>
<tr>
<td>Cry-C10</td>
<td>2.80 ± 1.34</td>
<td>1.40 ± 1.13 ($)</td>
</tr>
<tr>
<td>Cry-C14</td>
<td>1.30 ± 0.78</td>
<td>1.87 ± 0.93 ($)</td>
</tr>
<tr>
<td>Con-S</td>
<td>0.32 ± 0.39</td>
<td>0</td>
</tr>
</tbody>
</table>

Uni-S7, Uni-S10 and Uni-S14 are scrotal testes of the unilateral testis removal group on 7, 10 and 14 days after the operation, respectively; Cry-S7, Cry-S10, Cry-S14 are scrotal testes of the unilateral cryptorchidism group on 7, 10 and 14 days after the operation, respectively; Cry-C7, Cry-C10, Cry-C14 are abdominal testes of the unilateral cryptorchidism group on 7, 10 and 14 days after the operation, respectively; Con-S is the pooled data of the sham-operation for there is no significant difference between the day points. Numbers were shown as mean ± SD. Notes: ‘$t$’ indicates $P < 0.01$ compared with Con-S by Dunnett $t$ (2-sided) test after one-way ANOVA analysis. # means no data.
negative in apoptosis detection (Fig. 1).

2.2 The paralleled distributions of Uchl1, Jab1 and p27\textsuperscript{kip1} in spermatocytes revealed by immunohistochemistry

In the Row Con in Fig. 2, we can find that in controls to immunohistochemical detection (IHC) non-specific reaction was not detectable. In Row Uchl1, in sham-operated mouse control (column Con-S14), Uchl1 signal was positive specifically in some spermatogonia and Sertoli cells, and in step 10-11 spermatids, but nearly no positive signal was found in spermatocytes. In the scrotal testicular sections of 10 days after injury (Cry-S10, picture not included), the distribution pattern of Uchl1 was found similar to the control, and as Kwon et al. \cite{6} reported. However, in the abdominal testes (Cry-C10, Cry-C14), the positive immunohistochemical staining of Uchl1 protein was found abundant in apoptotic spermatocytes and some of them seemingly concentrated in the nuclei. In Row Jab1 (Fig. 2), in the control (Column Con-S14), Jab1 protein was observed in step 10-11 spermids, and sporadically in some spermatogonia and Leydig’s cells. But in the unilateral artificial cryptorchid on day 10 and 14 after operation (Cry-C10 and Cry-C14), the intense staining for Jab1 was detected in a small quantity of spermatocytes with apoptotic appearances and even sloughing from the seminiferous epithelium. As for p27\textsuperscript{kip1}, in the physiological conditions, it was mainly located in the Sertoli cells (Fig. 2, Row p27\textsuperscript{kip1}), but in artificial cryptorchids on day 10 and day 14 after operation (Cry-C10 and Cry-C14), p27\textsuperscript{kip1} emerged abundantly in some spermatocytes.
matocytes of apoptotic appearance as was shown for Uch1l and Jab1. However, in most MC, stainings of Uch1l, Jab1 and p27kipl were not observed, though MC constitutes a large part of the loss of spermatogenic cells resulted from cryptorchidism, especially at the later stage (i.e. Cry-C10 and Cry-C14 relative to Cry-C7).

2.3 The binding of Uch1l and Jab1 in the protein extracts of adult mouse testes in vitro
The GST-Uch1l affinity, or pull-down analysis, confirmed the possible interaction between Uch1l and Jab1 in adult mouse testes (Fig. 3), as in the tumor cells [12]. The GST-Uch1l pulled out the Jab1 from protein extracts of adult mouse testes, and GST did not (Fig. 3), suggesting that the Jab1 bound specifically to Uch1l. But we found the Jab1 had two bands in the Western blotting analysis of adult mouse testes protein extracts (loading control), and the band detected in the Uch1l pull-down located between the two bands in control.

2.4 The co-localization of Uch1l, Jab1 and p27kipl in cryptorchids detected by immunofluorescent staining
Since adding corresponding antigens could eliminate the positive signals, nonspecific immunoreactive binding was excluded (Fig. 4 Row Con). Different immunofluorescent color labeling exhibited the overlapped co-localization of Uch1l and Jab1 in the apoptosis-like spermatocytes (Fig. 4 Row Jab1 + Uch1l). The yellow fluorescence in the overlapped picture represented for the positional coincidence of Uch1l and Jab1. Similarly, the overlapped distribution of p27kipl and Jab1 were detected in the spermatocytes with apoptotic appearance (Fig. 4 Row Jab1 + p27). Scale bar, 50 µm.

3 DISCUSSION
The weight loss of unilateral experimental cryptorchid testis was induced by slightly elevated (3–4 °C) environmental temperature, since the endocrinological influences could be excluded in the present experiment according to the result that the contralateral scrotal testis was not inflicted. The spermatogenic cell removal in the cryptorchid testis was found to be caused by the initial occurrence of AC and the formation of MC subsequently. The process of large-scale spermatogenic cell loss in the initial stages was facilitated by the formation of MC, which stained negative for apoptosis [19]. Those two main removal ways of spermatogenic cells were determined by ordinary HE staining and TUNEL labeling in the present paper. The AC appeared earlier (in Cry-C7) and MC was observed later (in Cry-C14), so it can be deduced that the apoptosis was the upstream response to heat-stress, and MC was successive changes. Maybe AC induced Sertoli cells to phagocyte spermatogenic cells or spermatogenic cells fused by their own to form MC.
We found that Uchl1 concentrated into spermatocytes with apoptotic appearance to respond the abdominal hyperthermia. The accumulation of Uchl1 in the spermatocytes was caused probably by the blockade of degradation or the up-regulation of its expression. This discovery will favor the explanation for the involvement of Uchl1 in the apoptosis of spermatogenic cells. The apoptosis in experimental unilateral cryptorchid testis was proved to be related to Uchl1 for its homogenous deletion (gad mice) abrogated the apoptotic response of spermatogenic cells to the heat stress [8]. Furthermore, the testes of Uchl1 transgenic mice showed the arrest of spermatogenesis by massive spermatogenic cell death, leading to male sterility, suggesting that overexpression of Uchl1 promoted the apoptosis of spermatocytes [11, 20]. So the apoptosis induced by the elevated abdominal temperature was possibly mediated by the Uchl1 abnormal increase in spermatocytes. We found that cryptorchidism-induced spermatogenic cell loss was due to two different pathological processes, the initial occurrence of AC and the formation of MC, it is more important that the spermatogenic damage from cryptorchidism in response to intraperitoneal heat was attributed to meiotic stage, or the enrichment of Uchl1 in the protein spermatocytes.

About the mechanisms that Uchl1 protein involved in apoptosis, Kwon [11] proposed that Uchl1 promoted apoptosis via the maintenance of monoubiquitin levels to participate in the polyubiquitination of apoptosis related proteins. The levels of anti-apoptotic proteins, such as Bcl-2, Bcl-XL, and XIAP, were up-regulated respectively in the cryptorchid testes of gad mice, analogous to Uchl1-null mice, compared with those in wild type mice [8]. Moreover, Kwon et al. suggested a possibility that Uchl1 released the free ubiquitin monomers by hydrolyzing ubiquitin carboxyl-terminal derivatives, and the anti-apoptotic proteins were degraded via the ubiquitin dependent hydrolytic pathway [8]. In the present paper, we found that Uchl1 concentrated into spermatocytes of apoptotic appearance together with Jab1 and p27\(^{kip1}\). Additionally, by in vitro protein affinity with recombinant Uchl1, we found, similarly as in the case of tumor cells [12], Uchl1 can bind specifically to Jab1, suggesting other possible pathways of Uchl1 involvement in the apoptosis of spermatocytes.

A novel mechanism underlying Uchl1 involvement in the apoptosis induced by the heat-stress of cryptorchidism, which in some degree is different from Kwon et al. [11], for the present paper discovered that Uchl1 bound to Jab1 to affect the p27\(^{kip1}\) degradation in spermatocytes. p27\(^{kip1}\) was associated with Jab1 to transport into the cytoplasm to degrade [12–15]. If the degradation of p27\(^{kip1}\) was inhibited, the meiotic progression would be arrested, and spermatogenic cells will turn to apoptosis.

Since we observed in this paper that in the normal adult mouse testes there was a strong expression of p27\(^{kip1}\) in all Sertoli cells, and no p27\(^{kip1}\) staining in spermatogenic cells, as reported, p27\(^{kip1}\) seems to play a role in the development of spermatocytes via Sertoli cells [16, 21]. In addition to that p27\(^{kip1}\) (CDKN1B) plays a key inhibitory role in regulating Sertoli cell proliferation during the testicular development, observations on the knockout mouse indicate that p27\(^{kip1}\) has a role in the regulation of spermatogonial proliferation, or apoptosis, and the onset of the meiotic prophase in preleptotene spermatocytes [16]. In this paper, we found p27\(^{kip1}\) increased in some spermatocytes, just paralleled to Jab1 and Uchl1, in response to heat-stress, and apparently concentrated in nuclei of apoptotic spermatocytes. Injection of purified p27\(^{kip1}\) into Xenopus oocytes potently inhibits the G2/M transition and activation/dephosphorylation of the maturation promoting factor (MPF, p34cdc2/cyclin B complex) kinase associated with germinal vesicle breakdown (GVBD) induced by progesterone or insulin [22]. Thus, as shown in the present paper, p27\(^{kip1}\) in spermatocytes can directly inhibit the meiotic advancing. But in the other way, the massive apoptosis in spermatogenic cells of Skp2\(^{−/−}\) animals is attributable to the anti-proliferative effect of p27\(^{kip1}\) accumulation [23]. So p27\(^{kip1}\) may mediate the Uchl1-involved apoptosis directly through its abnormal increase in spermatocytes in response to heat-stress.

In addition, Jab1 is essential for E2F1-mediated apoptosis induction, and as an important transcription factor, E2F1 induces apoptosis through up-regulating pro-apoptotic genes such as ARF, p73, APAF1, Casp3, Casp7, and Chk2, and down-regulating anti-apoptotic genes of Traf2 and Mcl1, reviewed by Hallstrom et al. [24]. So Jab1 possibly mediates the Uchl1-involved apoptosis independent of p27\(^{kip1}\).

In conclusion, under the physiological conditions, Uchl1, Jab1 and p27\(^{kip1}\) were not expressed in spermatocytes, but in response to the thermal stimulus of experimental cryptorchidism, the three kinds of proteins occurred simultaneously in apoptosis morphological spermatocytes. The initial apoptosis of spermatocytes and subsequent formation of MC induced by
heat-stress are two main ways of spermatogenetic cell loss in cryptorchid testes, but only the former was relat-
ed to Uchl1. Uchl1 occurred abnormally in the sper-
matocytes in response to abdominal hyperthermia, and
in association with Jab1 and p27kip1, can be used to
specify the mechanisms of Uchl1 involvement in the
heat-stress-induced apoptosis of spermatocytes.

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